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Fine-mapping of *SRT7* for short roots and identification of its candidate in rice

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Three allelic short root mutants were identified by screening mutants with defective root elongation of the rice *japonica* cultivar Nipponbare mutant library generated via ^{60}Co γ-ray irradiation mutagenesis. These mutants, designated *srt7-1* (*short root 7-1*), *srt7-2* and *srt7-3*, respectively, had an extremely short seminal root, adventitious roots and lateral roots. Histological observation revealed the cell length of *srt7* mutant roots was significantly shorter than that of wild-type roots. Genetic analysis indicated the short root phenotype was controlled by a single recessive nuclear gene. The *SRT7* gene was mapped to a 20-kb interval between the markers STS6 and STS7 on chromosome 4 by a map-based cloning method. Sequencing of the six predicted genes in this region found that all of the three allelic mutants contained a 1-bp or 2-bp deletion in the same gene encoding a putative membrane-bound endo-1,4-β-glucanase. The *SRT7* gene was expressed ubiquitously, with higher levels of transcript accumulation in roots at different developmental stages. However, no difference was found in the *SRT7* transcription level between the mutant and wild type. Collectively, these results indicate the endo-1,4-β-glucanase encoding gene (LOC_Os04g41970) is likely the candidate for *SRT7* that functions posttranscriptionally in rice root elongation.

rice, short root, endo-1,4-β-glucanase, map-based cloning

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Roots are an essential vegetative organ of plants that contribute to water and nutrient uptake and provide anchorage to the soil. The root system of rice (*Oryza sativa*) consists of an embryonic seminal root and postembryonic adventitious roots and lateral roots [1]. Root elongation depends on two successive processes: cell elongation and cell proliferation [2]. Blocking either of the processes can inhibit the normal growth of roots and result in short roots. Many short-root mutants, such as *rm1*, *rm2*, *rrl1*, *rrl2*, *srt5* and *srt6*, have been isolated [3–7]. Among these mutants, elongation of the seminal root, adventitious roots and lateral roots of the *srt5* mutant were all inhibited. Other mutants only showed a short seminal root. A trait shared by all of these mutants is reduced cortical cell elongation [8]. The rice root growth-inhibiting mutant (*rt* mutant) has a short-root phenotype, which is caused by disturbance of the normal formation of root epidermal systems, and the recessive *rt* gene is located between RFLP markers C335 and R3351 on the long arm of chromosome 4 [9]. Ning et al. [10] isolated a short-root mutant (*ksr1*) from the Kasalath mutant library; the length of the seminal root of *ksr1* is only 20% of that of the wild type. Genetic analysis indicated the short-root phenotype of *ksr1* is controlled by a recessive nuclear-encoded gene, which was mapped to a 155-kb interval between the indel marker 4-24725K and simple sequence repeat (SSR) marker RM17182 on chromosome 4 [10].

To date, four short-root genes, *OsGNA1*, *GLR3.1*, *OsCyt-inv1* and *SPR1* have been cloned [11–14]. *osgna1*, a rice mutant generated from T-DNA insertion, has a short seminal root, adventitious roots and root hairs when grown

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at a low temperature (25°C). The *OsGNA1* gene encodes a glucosamine-6-P acetyltransferase involved in UDPN-acetylglucosamine biosynthesis. UDPN-acetylglucosamine is essential for glycosylation of many secreted and membrane-associated proteins in eukaryotes. Loss of function of *OsGNA1* disrupts microtubule orientation and blocks processes involved in root elongation [11]. *GLR3.1*, another rice T-DNA insertion mutant showing a short seminal root, adventitious roots and lateral roots at the seedling stage, can produce roots of normal length at later developmental stages [12]. *Oscyt-inv1*, a rice short-root mutant generated by EMS mutagenesis, has a seminal root, adventitious roots and lateral roots only 15%–20% of the length of those of the wild type. The *OsCyt-inv1* gene encodes an alkaline/neutral invertase that cleaves sucrose into glucose and fructose irreversibly. Exogenously supplied glucose rescues the root growth defects of the *Oscytinv1* mutant. These results show normal sugar metabolism is necessary for root development [13]. The rice short-root mutant *osspr1* is derived from the *indica* cultivar Kasalath. Elongation of the seminal root, adventitious roots and lateral roots of the *osspr1* mutant is inhibited sharply, especially for lateral roots. The length of lateral roots in 7-d-old seedlings of the *osspr1* mutant is only 12% of that of the wild type. The *OsSPR1* gene encodes a novel mitochondrial protein with an Armadillo-like repeat domain involved in iron homeostasis in rice [14].

To better understand the genetic control of root elongation in rice, in this study three allelic mutants that showed defective root elongation were identified, fine-mapping of the causal gene was conducted, and the candidate gene was predicted.

1 Materials and methods

1.1 Plant materials and growth conditions

Three rice short-root mutants (*srt7*-1, *srt7*-2 and *srt7*-3) were screened previously from the *japonica* cultivar Nipponbare mutant library generated via ^{60}Co γ-ray irradiation mutagenesis. For phenotype analysis, plants of the wild type and *srt7*-1 mutant were planted on floating nets in a black plastic container in nutrient solution and grown in QHX-350BS-III growth chambers at 25/22°C (day/night) with a 12-h photoperiod for 10 d.

1.2 Microscopic analysis

Lateral roots and root hairs were observed and photographed using a Nikon SMZ1000 stereomicroscope with a color CCD camera. For microscopic analysis, the base of the seminal root was treated with clearing reagent (60 mL H_2O, 160 g chloral hydrate and 20 mL glycerol) for 5 min. The samples were observed and photographed with a Nikon Eclipse Ti-S microscope with a color CCD camera.

1.3 Genetic analysis and construction of F_2 mapping populations

The short-root mutants *srt7*-1, *srt7*-2 and *srt7*-3, as the female parents, were crossed to the *japonica* cultivar Nipponbare and an *indica* cultivar Kasalath. The phenotype of F_1 and F_2 seedlings was investigated and F_2 mapping populations were generated by crossing *srt7*-1, *srt7*-2 and *srt7*-3 with Kasalath.

1.4 Gene mapping experiments and molecular marker development

Twenty F_2 individuals that showed the mutated phenotype of *srt7-1* were used for primary mapping and 2756 F_2 mutated individuals were analyzed for fine-mapping of the *srt7-1* locus. PCR-based sequence-tagged site (STS) markers were developed from an alignment of genomic sequences of *japonica* and *indica* rice (NCBI).

1.5 Sequence analysis and candidate gene identification

Candidate genes were predicted in the target region based on information in the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The PCR products of these genes were amplified from *srt7-1* genomic DNA by PCR using KOD-plus Polymerase, ligated to the Promega T-easy Cloning Vector, and sequenced (Shanghai Genomics Company). Sequence data were compared with the Nipponbare sequence database (NCBI) using DNAstar software (http://www.dnastar.com).

1.6 Semiquantitative RT-PCR analysis

Total RNA was isolated from rice tissues using the RNAgents total RNA isolation system (Takara, Dalian, China). RNA samples treated with DNAase I were reverse-transcribed by M-MLV reverse transcriptase (Promega). The following gene-specific primers were used for cDNA amplification: 5′-GCATCTCTCAGCACATTCCA-3′ and 5′-CTGGTACCCTCATCAGGCAT-3′ for *OsActin* (an internal control), and 5′-AGGCCCTCCTCTTCTTCAAC-3′ and 5′-GCTGAGCATTGTCATGGAGA-3′ for *SRT7*.

2 Results

2.1 Characterization of the *srt7* mutants and genetic analysis

Three allelic mutants were identified from screening of the *japonica* cultivar Nipponbare mutant library. These mutants had similar phenotypes, such as dwarfism and extremely short seminal roots, adventitious roots and lateral roots (Figure 1), and were designated temporarily *short root7-1*

(*srt7*-1), *short root7-2* (*srt7*-2) and *short root7-3* (*srt7*-3). The plant height (6.34 ± 0.31 cm) of 10-d-old *srt7-1* mutant seedlings was significantly shorter than that of the wild type (7.83 ± 0.23 cm). The length of the seminal root (1.23 ± 0.13 cm), the average length of the 4 longest adventitious roots (0.97 ± 0.07 cm), and the average length of the 4 longest lateral roots on the seminal root (0.23 ± 0.02 cm) were significantly shorter in comparison with those of the wild type (7.47 ± 0.33, 5.28 ± 0.52 and 1.47 ± 0.19 cm, respectively) (Table 1). The number of lateral roots in the *srt7* mutants was reduced, but the number of adventitious roots in the mutants was slightly increased, compared to those of wild-type plants (Table 1). In addition, root hairs were not obvious in the *srt7* mutants, whereas many root hairs were present on the seminal root of wild-type plants (Figure 1(b) and (c)).

Histological analysis showed that the cell length in the maturation zone of seminal roots of *srt*7 mutants was reduced and the cell shape was irregular (Figure 1(d) and (e)). These results indicated the embryonic and postembryonic roots and root hair development were arrested in the mutants. The *srt7* mutants showed a short-root phenotype throughout the plant life cycle but survived to maturity and produced seeds.

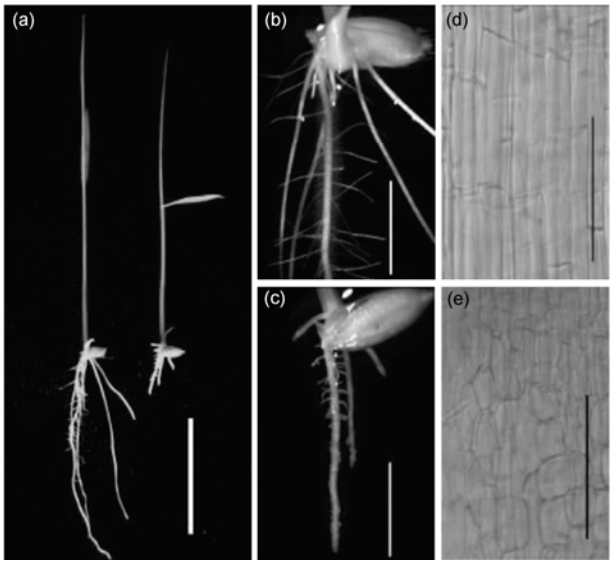

Figure 1 Phenotypic characteristics of *srt7-1* mutants. (a) Phenotypes of 6-d-old seedlings of the wild type (WT; Nipponbare; left) and *srt7-1* mutant (right) grown in growth chambers with a 12-h photoperiod under 25/22°C (day/night). (b) and (c) Root phenotypes of 6-d-old seedlings of the WT (b) and *srt7*-1 mutant (c). (d) and (e) Cortical cells in the maturation zone of seminal roots in the WT (d) and *srt7-1* mutant (e). Bar = 3 cm (a), 1 cm (b, c), and 50 μm (d, e).

To investigate inheritance of the short-root phenotype, the *srt7*-1 mutant, as the female parent, was crossed with the *japonica* cultivar Nipponbare and *indica* cultivar Kasalath. The root length was normal in one-week-old F_1 seedlings from both crosses. The F_2 population of *srt7*-1 × Nipponbare showed segregation of the WT and short root phenotypes (WT: mutant, 159:38), which fitted well a ratio of 3:1 ($\chi^2 = 3.128596 < \chi^2_{0.05} = 3.84$, $P > 0.05$). A similar segregation ratio was observed in the F_2 population of *srt7*-1 × Kasalath (WT: mutant, 257:84; $\chi^2 = 0.0088 < \chi^2_{0.05} = 3.84$, $P > 0.05$), which indicated the short-root phenotype was controlled by a single recessive nuclear gene.

2.2 Fine-mapping of the *SRT7* gene

Genetic mapping of the *SRT7* gene was performed using the F_2 population generated from a cross between *srt7-1* and Kasalath. For primary mapping, 20 F_2 individuals showing the mutant phenotype were analyzed using 120 SSR markers distributed with 10–15 cM intervals on 12 chromosomes. The *SRT7* gene was mapped in the region between the RM241 and RM564 markers on chromosome 4. Seven STS markers were developed by comparison of the genomic sequences of *indica* and *japonica* rice (Table 2). The genotypes of 2756 F_2 short-root mutants were analyzed using these polymorphic markers. The genomic region that harbored the *srt7-1* locus was narrowed down to a 20-kb interval between the STS6 and STS7 markers in AL606627 (Rice Genome Research Program (http://rgp.dna.affrc.go.jp/), (Figure 2(a)).

2.3 Prediction of the candidate gene

Six hypothetical or expressed genes were located in the 20-kb interval identified by the Rice Genome Research Program, which comprised an endo-1,4-β-glucanase,

Table 1 Plant height and root parameters of 10-d-old seedlings of the wild type (WT) and *srt7* mutants[a)]

Material	Plant height (cm)	Seminal root length (cm)	Adventitious root number	Average adventitious root length (cm)[b)]	Lateral root number	Average lateral root length (cm)[c)]	Cortical cell length (μm)[d)]
WT	7.83±0.23	7.47±0.33	4.75±0.44	5.28±0.52	81.00±2.00	1.47±0.19	53.25±5.85
srt7-1	6.34±0.31	1.23±0.13	6.75±0.38	0.97±0.07	18.00±2.50	0.23±0.02	13.87±1.26
srt7-2	3.64±0.03	1.09±0.13	7.25±0.75	0.87±0.07	19.25±2.75	0.14±0.03	12.26±1.81
srt7-3	5.52±0.34	1.21±0.14	7.00±1.00	1.17±0.04	17.00±3.50	0.27±0.07	13.11±0.96

a) Values represent the mean ± SE (n = 4). b) The average of the four longest adventitious roots was determined for each plant, then the average for four plants was obtained. c) The average of the four longest lateral roots was determined for each plant, then the average for 4 plants was obtained. d) The cortical cell length in the maturation zone of seminal roots in 6-d-old WT and mutant seedlings was measured.

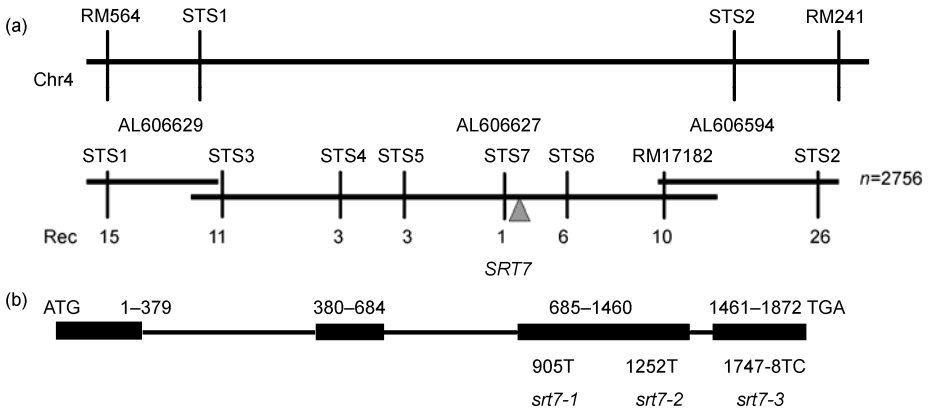

Figure 2 Fine-mapping and gene structure of the *SRT7* gene. (a) Fine-mapping of the *SRT7* gene on chromosome 4. The number of recombinants from 2756 F_2 mutant plants and the chromosomal positions of all markers are indicated. The triangle indicates the position of the *SRT7* gene. (b) Structure of the *SRT7* gene and the deletion mutations of three allelic mutants. Exons are indicated by a black box, whereas introns are indicated with a black line. Rec, recombinant.

Table 2 Polymorphic sequence-tagged site (STS) markers between the two parents[a)]

Marker name	Primer sequence forward (5′→3′)	Primer sequence reverse (5′→3′)	BAC
STS1	TTCTTGCTTTCCGTCTTGTT	ACTTTCTGCCGTACCGATT	AL606629
STS2	CAAGTGTAGCCTAGTGGTT	AGATGAAAGAAAGGGAGAT	AL606594
STS3	GCATACACTGTGCCTCATT	CAGCCTTGTTCTGTTCGTT	AL606627
STS4	TTTGAATGTGCACACTGCTG	GGCGCTATCGTTTCGTTTAT	AL606627
STS5	GGGAGCGAATTCTGTTTCTG	TTTTTCTTGCGGGTTTGTTC	AL606627
STS6	GACATTTGACACCCCCAAAC	TGGGTCCCACAGTATTTGTT	AL606627
STS7	CAGCTTTCGGAAAAGTTGGA	ACACACGGCTTCGTACGTG	AL606627

a) The two parents are the *japonica* cultivar Nipponbare and indica cultivar Kasalath.

riboflavin synthase and four hypothetical proteins. Genomic sequences of all 6 genes were sequenced and analyzed. A 1-bp deletion in exon 3 of an endo-1,4-β-glucanase (Loc_Os04g41970; *OsGLU3*) gene in the *srt7-1* mutant was detected, which created a frame-shift mutation and caused premature termination of protein synthesis. In addition, a 1-bp deletion and 2-bp deletion occurred in the endo-1,4-β-glucanase gene of the *srt7-2* and *srt7-3* mutants, respectively (Figure 2(b)), which provided additional evidence that *OsGLU3* is very likely the candidate gene for *SRT7*.

2.4 Expression pattern of *SRT7* in the wild type and *srt7* mutants

The expression pattern of *SRT7* was investigated in different tissues at different developmental stages of wild-type plants using semiquantitative RT-PCR. The *SRT7* gene was expressed constitutively in the root, stem, leaf and panicle, but higher levels of transcript accumulation were always detected in the roots of wild-type plants at different developmental stages (Figure 3(a)). No significant difference was observed between *SRT7* mRNA levels in mutants compared to that of wild-type plants (Figure 3(b)). These results indicated that the deletion in *srt7* does not affect its mRNA transcription.

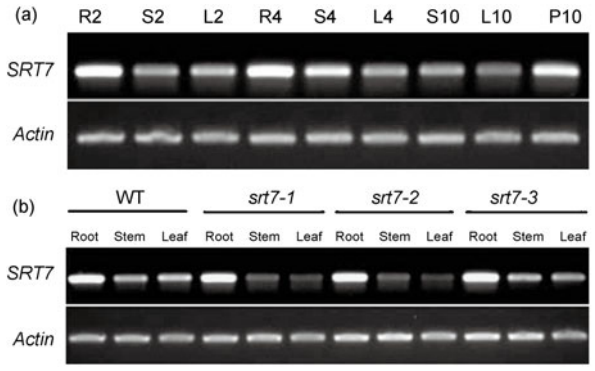

Figure 3 Expression of *SRT7* in the wild type (WT) and *srt7* mutants. (a) Expression pattern of *SRT7* in WT. cDNA was amplified from the root (R), stem (S), leaf (L) and panicle (P) of 2-, 4- and 10-week-old plants. (b) *SRT7* expression in the root, stem and leaf of 10-d-old WT and *srt7* mutants. Semiquantitative RT-PCR was performed for *SRT7* (30 cycles) using *OsActin* as an internal control (27 cycles).

3 Discussion

Among known rice short-root mutants, the *srt7* mutants show a similar phenotype to that of *rt*, *ksr1* and *Oscyt-inv1* mutants [9,10,13]. The *SRT7*, *RT* and *KSR1* genes should be allelic because not only do *srt7-1*, *rt* and *ksr1* share a similar phenotype but the three genes are located in the same region

of the genome. The only difference between these mutants is their genetic background; *ksr1* is derived from the *indica* cultivar Kasalath and *srt7* from the *japonica* cultivar Nipponbare. Elongations of all *srt7* mutant roots, including the seminal root, adventitious roots and lateral roots, were inhibited severely throughout the entire plant life cycle. This differed from other rice short-root mutants, such as the *GLR3.1* mutant, in which the short-root phenotype was only observed at the seedling stage [12].

The prediction of an endo-1,4-β-glucanase (*OsGLU3*) gene as the candidate for *SRT7* was supported by sequence data for two other allelic root mutants (Figure 2(b)). Endo-β-1,4-D-glucanase, which functions to hydrolyze β-1, 4-linkages on unsubstituted glucose residues, is involved in the formation of cell wall structure [15]. Many studies have showed that endo-1,4-β-glucanase plays an important role in cell elongation. In *Arabidopsis thaliana*, mutation of KOR, a membrane-bound endo-β-1,4-glucanase, causes dwarfism [16,17]. *CEL1*, a gene that encodes an endo-1,4-β-glucanase, is suggested to be associated with elongation of the root and shoot in *A. thaliana* [18,19]. At least 15 endo-1,4- β-glucanases are present in the rice genome [20]. *OsGLU1,* the first functionally known endo-1,4-β-glucanase, plays an important role in internode elongation and assembly of cell wall components in rice [21]. Loss of function of *OsGLU1* causes a reduction in cell elongation and plant height, a decrease in cellulose content, and an increase in pectin content. Previous studies showed that endo-1,4-β-glucanase is essential for cell elongation in both dicotyledons and monocotyledons and allows the confident prediction of *OsGLU3*, a homolog of *OsGLU1*, as the candidate gene for *SRT7*. The finding that three allelic root mutants with different positional mutations in the coding sequence had almost identical phenotypes indicated very strongly the importance of protein integrity for its biological function. Clearly, these allelic mutants should be valuable for further studies on the genetic basis of root elongation in rice.

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